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The major reverse-transcriptase-incompetent splice variant of the human telomerase protein inhibits telomerase activity but protects from apoptosis

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Abstract

hTERT (TERT), the catalytic protein subunit of telomerase, is subjected to numerous alternative splicing events, but the regulation and function of these splice variants is obscure. Full-length hTERT includes conserved domains that encode reverse transcriptase activity, RNA binding and other functions. The major splice variant termed α+β− or β-deletion is highly expressed in stem and cancer cells, where it codes for a truncated protein lacking most of the reverse transcriptase domain but retaining the known RNA binding motifs. In a breast cancer cell panel, we found that β-deletion was the hTERT transcript that was most highly expressed. Splicing of this transcript was controlled by the splice regulators SRSF11, HNRNPH2 and HNRNPL and the β-deletion transcript variant was associated with polyribosomes in cells. When ectopically overexpressed, β-deletion protein competed for binding to hTR (TERC) RNA, thereby inhibiting endogenous telomerase activity. Overexpressed β-deletion protein localized to the nucleus and mitochondria and it protected breast cancer cells from cisplatin-induced apoptosis. Our results reveal that a major hTERT splice variant can confer a growth advantage to cancer cells independent of telomere maintenance, suggesting hTERT makes multiple contributions to cancer pathophysiology.

Keywords

Telomerase; hTERT; alternative splicing; apoptosis; breast cancer cells

INTRODUCTION

Telomerase is a highly regulated ribonucleoprotein (RNP) complex that adds TTAGGG DNA repeats to human telomeres. The human core telomerase complex contains a catalytic reverse-transcriptase protein subunit (hTERT) and telomerase RNA (hTR/hTERC; 1). In normal somatic cells, telomerase activity is generally undetectable or present at low levels (2). In contrast, telomerase activity is high in 80–90% of human cancers, consistent with the ability of these cells to maintain stable telomere lengths (3). In most human cancer cells, telomerase activity is limited by the level of reverse-transcriptase (RT) hTERT expression (4). While hTERT transcriptional regulation has been extensively studied, the regulation of hTERT through mRNA processing events is less well understood. One major mRNA processing regulatory mechanism is alternative splicing, which can both control transcript

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abundance and contribute to proteome diversity. TERT pre-mRNA is alternatively spliced in many species, ranging from plants to humans (5). To date, 14 human hTERT transcript variants have been detected (6), including the full-length RT competent form (RT+). In cells exhibiting telomerase activity, such as stem cells and tumor cells, several hTERT splice variants are co-expressed at significant levels (6–8). Understanding why these splice variants exist and how they are regulated is necessary to provide further insight into the roles of TERT in cancer biology.

The two most studied hTERT alternative splice variants encode predicted proteins lacking catalytically active RT and are generated by alternative splicing at two sites, the α and β site (9; Fig. 1). Skipping 36 nucleotides in exon 6 deletes the α site, while splicing at the β site (exon 7 and 8) results in a 183-nucleotide deletion and generates a transcript harboring a premature termination codon (PTC). Both α and β splicing events occur within the telomerase RT (Fig. 1); splicing at the α site removes most of RT motif A from the coding sequence, while splicing at the β site results in a truncated protein lacking the RT motifs B – E (RT−) and the C-terminal part of hTERT (10, 11). Splicing at either site can occur separately or in combination and generates either α+β+, α−β+, α+β−, or α−β− transcripts.

One of the most abundant hTERT splice variants besides the α+β+ variant is the α+β− form. This variant (hereafter called β-deletion) is expressed in cancer cells and in normal human tissues in developmental stage-specific patterns, suggesting that it may serve an important physiological function (7, 8, 12). In human fetal kidney, a developmental switch abolishes expression of α+β+ (RT+) hTERT mRNA, but maintains expression of the β-deletion variant. This switch correlates with loss of telomerase activity during gestation (8). Similarly, *all-trans* retinoic acid promoted differentiation of promyelocytic leukemia HL60 cells induced downregulation of α+β+ hTERT mRNA, stabilization of β-deletion mRNA, and a decrease in telomerase activity (13). In addition, β-deletion mRNA expression has been correlated with low telomerase activity in a variety of human tumors and cancer cell lines (14, 15).

Together, these observations suggest that alternative splicing of hTERT pre-mRNA to the β-deletion splice variant could negatively regulate telomerase activity. It is unknown, however, whether this variant is translated into protein and if the β-deletion protein inhibits telomerase. Furthermore, it is unknown whether the β-deletion protein might serve additional, non-telomeric functions in the cell, as has been demonstrated for RT+ hTERT (16–18). To answer these questions, we examined the translation potential of the β-deletion mRNA variant and tested the effect of overexpressing this isoform on telomerase activity and the ability of cancer cells to induce apoptosis.

MATERIALS AND METHODS

Cell lines

Jurkat, UM-UC-3 HeLa, HEK293T and GM847 cell lines were purchased from ATCC. Culturing conditions and the panel of 50 human breast cell lines used for this study are explained in Supplementary Methods.

Plasmids and Lentivirus

Lentivirus preparation, transduction and control scramble shRNA were described previously (19). The UPF1 shRNA target sequences was GAGAATCGCCTACTTCACT (20). Transduced cells were puromycin selected for 3 – 5 days. The β-deletion construct was generated as described (11, see Supplementary Methods for details). WT-hTERT, D868A-hTERT and β-deletion were subcloned into pcDNA3 (Invitrogen) or lentivectors to generate pHR'CMV-hTERT-IRES-puromycin constructs. N-terminal FLAG epitopes

(DYKDDDDK) were inserted with complementary oligonucleotides into each plasmid carrying an hTERT variant. For microscopy, β-deletion was cloned into pIC111 (21). All plasmids were verified by sequencing.

Polyribosome profiling

Extract preparation, polyribosome fractionation, RNA extraction was described in (22). RNA associated with ribosomes was reverse-transcribed and analyzed by qPCR using SRSF3 and hTERT variant specific primers (14, 23).

Light Microscopy

HeLa cells were transfected with pIC111-β-deletion using Fugene HD (Roche). 48 h post transfection, cells were stained with 10 μg/ml Hoechst 33342 and 50 nM MitoTracker Deep Red FM (Molecular Probes) for 20 min at 37°C. Live images were acquired in 0.5 μm increments using a Deltavision RT (Applied Precision) with a 100X/1.40NA PlanApo objective (Olympus). Images were deconvolved and Z-projected in Softworx (Applied Precision) then adjusted for brightness and contrast in FIJI (24).

Splicing reporter analysis

For splicing analysis, 0.5 μg of each splicing factor and pSpliceExpress-hTERT (25) were transfected into HEK293T cells with Lipofectamine (Invitrogen). 48 h post transfection, total RNA was extracted, followed by reverse-transcription with rat insulin exon 3 reverse primers followed by hTERT qPCR. Cloning of pSpliceExpress-hTERT and panel of splicing regulatory proteins are described in Supplementary Methods.

Quantitative RT-PCR

Specific primers used are listed in Supplementary Table 4. DNA standards were prepared as described in (26). Serial DNA dilutions were used to generate external standard curves by plotting the standard concentration expressed as copy numbers/reaction vs. the second derivative maximum Cp. Total RNA was extracted (RNeasy Mini Kit; Qiagen), followed by cDNA synthesis using 2 μg RNA, random primers and Superscript III (Invitrogen). cDNA was amplified in 10 μl reactions containing LightCycler 480 DNA SYBR Green I Master (Roche Applied Science) and 0.5 – 1 μM final concentration of each primer using a LightCycler 480 (Roche Applied Science). For cycling parameters, see Supplementary Methods. Transcript copy number were obtained for each transcript and normalized by dividing by copy numbers for the housekeeping gene GAPDH to facilitate inter-cell line comparison.

Telomerase activity

Relative telomerase activity (RTA) was determined by the real-time quantitative telomere repeat amplification protocol RQ-TRAP (27). PCR amplification was performed with a Light Cycler 480 (Roche Applied Science). For cycling parameters, see Supplementary Methods.

Telomere length

DNA was isolated with the Gentra Puregene Cell Kit (Qiagen), digested with Hph1 and Mnl1, fractionated by a 0.6% agarose gel, transferred to a Hybond XL membrane (GE Healthcare) and hybridized to a ^{32}P-labeled 5′-$(CCCTAA)_4$-3′ probe. Signals were detected by phosphorimaging and analyzed with ImageQuant (GE Healthcare) to calculate modal telomere length with respect to a 10 kb marker.

Immunoprecipitation and Western blotting

GM847 ALT cells were transduced with FLAG-hTERT lentivirus and IP-TRAP procedure was performed essentially as described in (28) with M2 anti-FLAG antibody (Sigma Aldrich) and GammaBind G sepahrose (GE Healthcare). RNA-IP was performed in RIPA buffer (50 mM Tris-Cl, pH 7.5, 1 % Nonidet P-40, 0.5 sodium deoxycholate, 0.05 % SDS, 1 mM EDTA, 150 mM NaCl, protease inhibitors) + RNAsin. RNA was extracted from anti-FLAG immunoprecipitates, reverse-transcribed and analyzed by qPCR. SDS-PAGE and Western blotting was performed with immunprecipitates or whole cell lysates using the NuPAGE system (Invitrogen) and Hybond-P PVDF membrane (GE Healthcare) according to the manufacturer's instruction. For antibodies, see Supplementary Methods.

Apoptosis measurement

Cells were transduced, puromycin selected and expanded for less than 2 weeks and plated at 5000 cells/well in 96 well plates. 16 h later, cells were treated with cisplatin for 48 h. Cell confluency was determined with the INCUCYTE Live-Cell Imaging System (Essen BioScience) prior to Caspase-3/7 activity measurement (#G8091, Promega).

Statistical analysis

RTA levels were log-transformed to improve the normality of the distribution. Pearson's correlation coefficients were calculated to assess linear relationships between the variables. Two-tailed P-values were calculated and were considered statistically significant when < 0.05. All analyses were performed using GraphPad Prism version 5.0b for Mac.

RESULTS

The major hTERT β-deletion splice variant escapes NMD and is found in polyribosomes

Nine of the 14 hTERT alternative splice variants, including the β-deletion variant, introduce a PTC into the reading frame (6). PTC-containing mRNAs are generally targeted to the nonsense mediated decay (NMD) pathway for rapid degradation, and experimental inhibition of the NMD pathway leads to stabilization and accumulation of such mRNAs (29). Whether an mRNA with a PTC is efficiently targeted and destroyed by NMD determines its translation potential. To assess the level at which the NMD pathway controls the degradation of the β-deletion variant compared with the α+β+ forms of hTERT, we measured the accumulation of hTERT mRNAs upon shRNA-mediated depletion of the NMD pathway component UPF1. We chose three diverse human cancer cell lines (Jurkat [T-cell lymphoma], UM-UC-3 [bladder cancer], BT-549 [breast carcinoma]) that all express the β-deletion variant as measured by RTqPCR (14; see primer location in Fig. 4A) and have telomerase activity, as measured by RQ-TRAP (27; Fig. 2A). Although UPF1 protein levels were knocked down by at least 90% in all three cell lines (Fig. 2C), NMD activity differed between cell lines and transcripts, as reported previously (30). β-deletion transcripts accumulated more in UM-UC-3 compared to BT-549 and Jurkat cells upon UPF1 depletion (Fig. 2B). α+β+ hTERT transcripts (of which the RT+, full-length mRNA is the most prominent), also accumulated upon UPF1 depletion, and to higher levels in UM-UC-3 versus BT-549 and Jurkat cells. This observation is not surprising, as other splicing events in the same mRNA molecule that introduce a PTC outside of the probed region could subject the α+β+ mRNA to NMD. Furthermore, cycloheximide treatment, which is a pleiotropic inhibitor of NMD, resulted in similar mRNA accumulation patterns (Supplementary Fig. 1). To control for NMD inhibition, we measured the accumulation of an established NMD target, an SRSF3 splice variant with a PTC (SRSF3 PTC+; 23; Fig. 2B). These results indicate that in BT-549 and Jurkat cells compared to UM-UC-3 cells, β-deletion transcripts

are relatively insensitive to NMD, leaving the opportunity for transcripts to escape the NMD pathway and, potentially, be translated.

To investigate whether the β-deletion transcripts are actively translated, we determined whether this mRNA transcript is associated with the translation machinery, namely polyribosomes, since mRNAs bound to polyribosome are highly likely to be translated. Polyribosome profiling was employed because antibodies are not available that reliably detect the very low endogenous levels of hTERT isoforms (unpubl. data). We performed polyribosome profiling on the Jurkat cell line because of its relatively high levels of both of hTERT mRNA (BioGPS; 31) and telomerase activity (32). Following sucrose gradient centrifugation, ribosome/polyribosome-containing fractions of Jurkat cell extracts were analyzed for the abundance of hTERT variant mRNAs and compared to the efficiently translated SRSF3 PTC- mRNA and the bona fide NMD targeted (and therefore poorly translated) SRSF3 PTC+ mRNA by RT-qPCR (Fig. 2D-F)(14, 23). As expected, given its relatively low transcript abundance, hTERT mRNA was present at low amounts in all the ribosome-containing fractions compared to SRSF3 mRNA (Supplementary Fig. 2). Seventeen percent of the input α+ hTERT transcripts were β-deletion (α+β−), and a similar fraction of β-deletion transcripts were associated with polyribosomes (18 and 15% β-deletion transcripts in fractions 8 and 9, respectively), indicating that most of the β-deletion mRNA transcripts are translated. In contrast, of the total input SRSF3 transcripts (PTC+ and PTC−), 15% were PTC+, and only 2–3% of the PTC+ SRSF3 transcripts associated with polyribosomes in fraction 8 and 9 (Fig. 2E), as expected for this NMD-targeted mRNA with low translational competence. These results indicate that the β-deletion splice variant, although containing a PTC, istranslated because it is associated with multiple (7+) ribosomes and is not depleted from polyribosomes like the *bona fide* NMD-targeted SRSF3 PTC+ mRNA. The similarity of the polyribosome-bound and total steady state ratios of β-deletion: α+β+ hTERT mRNAs suggests that both β-deletion andα+β+ hTERT mRNAs are translated in Jurkat cells, producing RT− and RT+ hTERT proteins, respectively.

To determine the cellular localization of the β-deletion protein isoform, we designed an β-deletion overexpression construct carrying a C-terminal FLAG/GFP tag (21). When this construct was expressed in HeLa cells, we observed a GFP signal in the nucleus and nucleolus. In addition, we detected cytoplasmic GFP colocalizing with mitochondria (Fig. 3, Supplementary Figure 3). These results indicate that the RT− β-deletion protein localizes to the nucleus and mitochondria, as was previously described for overexpressed full-length RT + hTERT protein (33).

hTERT β-deletion alternative splicing is regulated by SRSF11, hnRNPL and hnRNPH2

Alternative splicing is regulated both by cis-regulatory sequence elements and protein regulators. A multitude of splicing regulatory proteins work in combination and competition to control the final splicing outcome for an individual pre-mRNA (34). SR proteins and hnRNPs are protein regulators that either enhance or repress splice site recognition and result in exon skipping or inclusion. To further characterize the regulation of the β-deletion splice variant, we designed a splicing reporter minigene (25) encompassing hTERT exons 5 to 9 (Fig. 4A). We separately overexpressed a panel of splicing regulatory factors (see Methods). Overexpression of SRSF11 strongly increased β-deletion splice variant mRNA levels (Fig. 4B–C). Conversely, overexpression of hnRNPL or hnRNPH2 decreased β-deletion splice variant levels. Searches for SRSF11 binding sites within the reporter minigene identified two sites upstream of the 3′ splice site in intron 6, three sites upstream of the 3′ splice site in intron 8 and one site in exon 9 that matched or closely matched the SRSF11 binding site consensus sequence (35, see Supplementary Fig. 4). Interestingly, some hnRNPH2 binding sites overlapped with SRSF11 binding sites. As inclusion or exclusion of the β site depends on whether exon 6 is joined to exon 7 or exon 9,

respectively, utilization of the 3′ splice site of either intron 6 or 8 is central to this alternative splicing decision. Together, these data suggest that SRSF11 and hnRNPH2 compete for binding to these sites to stimulate either β site exclusion or inclusion.

The β-deletion protein can act as a dominant-negative telomerase activity inhibitor by sequestering hTR

The role of the β-deletion hTERT in human cancer cells is unknown. Given its nuclear localization (as described above) and high relative abundance of its mRNA (as described further below), we anticipated a possible role for the β-deletion isoform in regulating telomerase activity. The predicted β-deletion protein retains the known hTR-binding domains (36). To test whether the β-deletion protein binds hTR, we isolated RNA from immunoprecipitation-purified telomerase RNPs. FLAG-tagged RT+ WT-hTERT, RT− point mutant D868A-hTERT (37) and RT− β-deletion were overexpressed from a lentiviral vector in GM847 cells, which express hTR but not hTERT (38), thus allowing assembly of a telomerase RNP containing the lentivirally overexpressed hTERT protein. hTR co-immunoprecipitated with FLAG-WT-hTERT, FLAG-D868A-hTERT and FLAG-β-deletion at 213, 29 and 60 fold % input amounts over vector, respectively (Fig. 5A). As shown in Figure 5C, less FLAG-β-deletion and FLAG-D868A protein immunoprecipitated than FLAG-WT-hTERT protein, indicating that the hTR was co-Immunopreciptated with the FLAG-β-deletion isoform relatively efficiently. We confirmed this result in a biological repeat of this experiment and in which we detected the co-immunoprecpitated hTR with a primer pair binding to a different region in hTR (Supplementary Figure 5). We conclude that the β deletion protein can bind efficiently to hTR.

We next tested whether β-deletion telomerase can elongate a telomeric DNA by measuring telomerase activity of immunoprecipitated telomerase RNPs. Immunoprecipitated FLAG-WT-hTERT showed an approximately 15,000 fold higher relative telomerase activity (RTA) signal over vector, while immunoprecipitated FLAG-β-deletion or FLAG-D868A showed essentially background RTA levels relative to vector (Fig. 5B). This result confirms that the β-deletion protein-containing telomerase does not possess telomerase activity, as expected due to the lack of RT motifs B–E and as reported previously (10, 11).

The above results indicate that the β-deletion can bind hTR but does not possess telomerase catalytic activity. Therefore, we predicted that the β-deletion should act as a dominant-negative inhibitor of endogenous telomerase activity. To test this hypothesis, we overexpressed β-deletion in UM-UC-3 bladder cancer cells. In this cell line, the level of hTR, rather than hTERT, is limiting for telomerase activity (40). Therefore, as expected for this cell line in which hTR is limiting, WT-hTERT overexpression did not alter RTA levels significantly compared to vector control, while hTR overexpression increased telomerase activity (Fig. 5D). In contrast to full-length hTERT overexpression, overexpression of either β-deletion or D868A reduced RTA levels in UM-UC-3 cells by 5 and 28 fold, respectively, compared with the endogenous levels. The dominant-negative effect of β-deletion overexpression could be partially rescued by simultaneous overexpression of hTR. This result is consistent with β-deletion competing with endogenous full-length hTERT for hTR binding, yet not having a dominant negative effect on the endogenous functional hTERT in the UM-UC3 cell line when hTR is no longer limiting. As expected, telomerase activity was not rescued in the D868A transduced cells that received the hTR construct, consistent with the established dominant-negative effect of this mutant. We further confirmed that the effect of the different constructs is attributable to relative expression levels of the hTR and hTERT constructs by measuring the RNA and protein levels of overexpressed constructs using RT-qPCR (Fig. 5D) and Western blot (Supplementary Fig. 6). While WT-hTERT, β-deletion and D868A mRNA levels and corresponding WT-hTERT and D868A protein levels were similar, β-deletion protein levels were slightly lower. We conclude that when hTR is

limiting, the β-deletion protein can act as a dominant-negative inhibitor of telomerase by sequestering hTR from the catalytically active hTERT. However, the greater dominant-negative effect of the full-length D868A mutant compared with β-deletion did not permit any conclusions as to whether D868A inhibited telomerase more due to higher expression levels or by mechanisms other than hTR sequestration.

The β-deletion mRNA is highly expressed in breast cancer cell lines

Although β-deletion transcripts are known to be expressed in a variety of cancer tissues and cell lines (14, 15, 26), few studies have rigorously quantified the abundance of this variant across a large set of cell lines or tumors. We therefore quantified the mRNA expression levels of the four hTERT α/β splice variants in a panel of 45 human breast cancer and five non-malignant human breast cell lines (Supplementary Table 1) by RT-qPCR (14). Steady-state levels of the total α/β splice variant mRNAs differed widely between the 50 cell lines (Fig. 6A–D). Across the panel, the α+β+ and β-deletion transcripts together constituted over 90% of all variants (on average ~47% each; Supplementary Table 2). The percentage of β-deletion transcripts relative to the total hTERT mRNA ranged from 21 % to 79 % among individual lines (Fig. 6A). Hence, in individual cell lines, the proportion of RT− β-deletion splice variant mRNA correlated inversely with the proportion of RT+ α+β+ mRNA. In contrast, the α−β+ and α−β− variants together accounted for only 0.4% and 6% average total hTERT transcripts, respectively. Thus, α− hTERT variants are weakly expressed, while on average β-deletion variants account for about half of the total hTERT mRNAs in these cell lines.

Expression of hTERT splice variants correlates with telomerase activity and breast cancer subtype

Given the dominant-negative effect of β-deletion overexpression on telomerase activity and the high levels of β-deletion mRNA in cancer cells, we tested two endpoints of possible dominant-negative action of β-deletion in the breast cancer panel: telomerase activity and telomere length.

RTA was measured by RQ-TRAP and ranged more than 300 fold across the 50 cell lines - from 4 to 1285 RTA units (Fig. 6B). As telomerase activity depends on both hTR and hTERT protein (41), we also quantified the levels of hTR RNA across the panel. All 50 cell lines expressed hTR, with expression levels ranging 100 fold between the lines (Supplementary Fig. 7). Telomere length was determined by measuring telomere restriction fragment (TRF) size distribution by Southern blot of DNA extracted from each cell line. TRF modal lengths ranged from 867 to 9,071 bp (Fig. 6C). Telomere lengths were stable over time, showing no significant change after 20 continuous passages in six randomly selected cell lines (Supplementary Fig. 8).

Pearson correlations (Supplementary Table 3) revealed that log(RTA) positively correlated with TRF sizes across the panel ($r = 0.487$, $P < 0.0001$, see also Fig. 6D), suggesting that higher telomerase activity generally results in longer telomeres. Log(RTA) levels were slightly negatively correlated with hTR levels ($r = -0.300$, $P = 0.034$). Minimally, this indicates that hTR is not a limiting factor for telomerase activity in these cells. The relative level of α+β+ hTERT mRNA correlated positively with log(RTA) ($r = 0.325$, $P = 0.021$). Conversely, the relative level of the β-deletion variant correlated negatively with log(RTA) ($r = -0.285$, $P = 0.045$), as has been shown previously in melanoma cell lines and lung cancer (14, 15). TRF lengths did not correlate significantly with either hTERT splice variant expression or hTR expression. This finding suggests that telomere length is determined by factors other than hTERT alternative splicing in these cell lines. Our correlation data, together with the finding that experimentally overexpressed β-deletion can inhibit

telomerase activity and bind hTR in cell extracts, suggests that endogenous β-deletion may limit telomerase activity by competing with the catalytically active hTERT protein for hTR.

To understand possible roles of β-deletion in cancer, we determined the relationship between hTERT variant mRNA levels, telomerase activity levels and clinically relevant phenotypes of the panel of 50 breast cancer and nonmalignant breast epithelial cell lines analyzed here. This panel models the transcriptional profiles of the luminal, basal, claudin-low and amplified HER2/ERBB2 receptor gene subtypes defined in primary tumor samples (42, 43). Expression of the RT+ hTERT α+β+ splice variant was statistically significantly higher in the basal subtype (which is associated with poor clinical outcome) and lower in the luminal subtype (which is associated with a better clinical outcome; Supplementary Fig. 9A). Conversely, expression of the RT− β-deletion variant was statistically significantly lower in the basal cell subtype and higher in the luminal subtype (Supplementary Fig. 9B). Furthermore, log(RTA) was statistically significantly higher in cells of the basal subtype then the luminal subtype (Supplementary Fig. 9C). None of the other hTERT splice variants, hTR or TRF size showed any significant differences between the subtypes (data not shown). Together, these results indicate that telomerase activity levels correlated with the major RT+ α+β+ and RT− β-deletion hTERT variants, suggesting that telomerase activity may be regulated by alternative splicing at the hTERT β site in these two breast cancer subtypes.

Overexpression of the β-deletion protein protects breast cancer cells from apoptosis

There is evidence that hTERT performs additional functions in the cell beyond telomere maintenance. hTERT and mTERT overexpression can protect normal and cancer cells from apoptosis (44–46), independent of catalytic activity (18, 44, 47). Therefore we tested the effect of β-deletion variant overexpression on apoptosis in three basal subtype breast cancer cell lines, BT549, HCC3153 and HCC1806, chosen for their different levels of β-deletion expression. Apoptosis was induced by treatment with cisplatin for 48 h, and activation of caspase 3/7 was monitored using a luminescence reporter assay. Cells overexpressing either the β-deletion or WT-hTERT protein showed significantly less caspase 3/7 activation compared to vector controls (Fig. 7A+B). These results indicate that β-deletion, like WT-hTERT, can protect cancer cells from cisplatin-induced apoptosis and cell death.

DISCUSSION

Although hTERT alternative splice variants were identified over a decade ago (9), the biological function of the major β-deletion variant remains unknown. Here we present evidence that the β-deletion isoform is translated and can act as a dominant-negative inhibitor of telomerase. Although the β-deletion transcript carries a PTC, it is present at high steady-state levels in our panel of breast cancer cell lines, is relatively insensitive to NMD and associates with polyribosomes, which is strongly indicative of its translation. We show that overexpressed β-deletion protein binds hTR RNA and suppresses telomerase activity. Simultaneous overexpression of hTR was sufficient to rescue this dominant-negative effect of β-deletion overexpression, suggesting that the dominant-negative effect is due to β-deletion binding hTR and either competing or otherwise interfering with full-length, RT+ hTERT function. Interestingly, we show that the RT− β-deletion, like full-length RT+ hTERT, can protect breast cancer cells from cisplatin-induced apoptosis. The β-deletion protein localized to the mitochondria, a potential cellular location where interference with the apoptosis pathway occurs.

Our results may explain why β-deletion transcript levels are negatively correlated with telomerase activity in both our study and those of other groups (14, 15). Given its truncated RT domain, our finding that β-deletion lacked telomerase activity is expected and supports previous *in vitro* studies (10). Alternative splicing of hTERT pre-mRNA into RT− β-

deletion appears to be at the expense of RT+ hTERT, and thus less catalytically active telomerase RNP is produced. Furthermore, translated RT− β-deletion protein inhibits catalytically active RT+ telomerase RNP. Thus, higher β-deletion transcript levels predict lower telomerase activity.

Another study failed to detect a decrease in endogenous telomerase activity upon overexpression of β-deletion in H1299 cells (11). This discrepancy could be explained by different expression levels of the β-deletion construct, since the prior study did not demonstrate β-deletion expression. The α−β+ hTERT variant has also been previously shown to act as a dominant-negative inhibitor of telomerase activity when experimentally overexpressed (11, 48). However, in the panel of breast cancer cell lines examined here, the abundance of α−β+ mRNA is low, while the abundant β-deletion mRNA approaches 50%. The inverse correlation between β-deletion and telomerase activity, and the low level of α −β+ mRNA makes it likely that in these cells, inhibition of telomerase is due to the β-deletion.

Although it has been shown that that elevated total hTERT mRNA level is linked to poor survival in breast cancer, most primers used to measure hTERT transcript levels by RT-qPCR are not designed to discriminate splice variants from full-length hTERT mRNA (49). We show that higher telomerase activity in basal subtypes is accompanied by a higher percentage of expression of RT+ α+β+ transcripts. Conversely, lower telomerase activity in luminal subtypes correlates with higher levels of the β-deletion variant. Our results indicate that hTERT subtype-specific splicing is one mechanism of differentially regulating telomerase activity in breast cancer subtypes.

Little is known about hTERT alternative splicing regulation. Depletion of the chromatin remodeling protein Brm in NCI-H1299 cells led to a decrease in hTERT α+β+ and β-deletion transcripts (50). Furthermore, Brm and the splicing factors PSF and p54/(nrb)/NONO were shown to bind to the hTERT gene close to exon 7, indicating the possibility of co-transcriptional splicing (50). We studied the splicing regulation of hTERT with a splicing reporter minigene, to improve quantitative detection of the subtle changes in variant expression when splicing regulatory factors are overexpressed. We demonstrate that β-deletion splicing is controlled by SRSF11, hnRNPH2 and hnRNPL. Few targets of SFRSF11 are known. Previously, SRSF11 was shown to stimulate the exclusion of tau exon 10, competing with Tra2β for binding to its target site (35). It will be interesting to test whether Tra2β, Brm, PSF or p54(nrb)/NONO also regulate hTERT β site splicing.

Why might cancer cells express such high levels of this catalytically inactive hTERT splice variant? We find that overexpression of β-deletion in three basal breast cancer cell lines significantly reduced the number of apoptotic events normally induced by cisplatin. However, the molecular mechanism by which full-length hTERT and β-deletion protect against apoptosis is unknown. Since we show that β-deletion, like hTERT, localizes to the mitochondria, one possibility is that hTERT and β-deletion interact with the apoptotic pathway at the mitochondria. Others have shown that hTERT overexpression enhances genomic stability and DNA repair (51). It is possible that both full-length hTERT and β-deletion protect against cisplatin-induced apoptosis by increasing the ability of the cells to repair cisplatin-induced DNA damage. Because both full-length RT+ hTERT and RT− β-deletion were capable of protecting cells from apoptosis, and it will be interesting to test which protein domains in hTERT are important for this property. The protection against apoptosis conferred by β-deletion suggests that the hTERT RT domain and C-terminus are not required for evading apoptosis. Further experiments and the generation of a β-deletion-specific antibody are needed to determine the molecular mechanism connecting the β-deletion to the apoptotic pathway. Even though β-deletion can interfere with endogenous

telomerase, this effect does not prevent sufficient telomere maintenance, because the breast cancer cell lines we tested maintain stable telomere lengths. Ultimately, cancer cells may need to achieve a balance between β-deletion expression that provides the benefit from protection against apoptosis and allowing for proper telomere maintenance. Although interfering with β-deletion splicing regulation might transiently increase resistance to apoptosis, such an approach may be incorporated into therapeutic tools to limit telomere maintenance and proliferation of cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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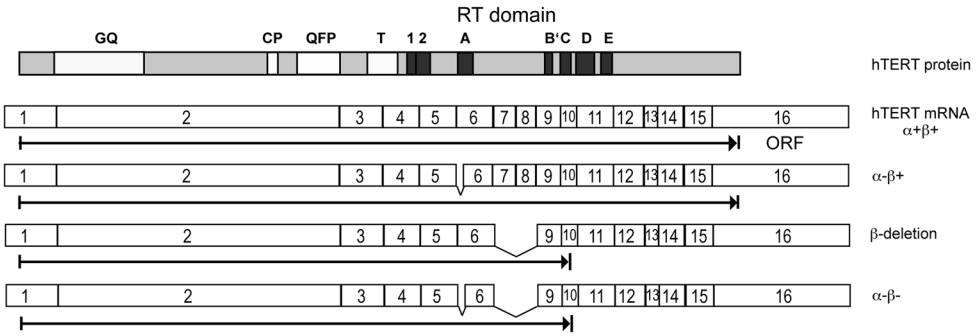

Figure 1. Schematic of hTERT α/βsplice variants

Top: hTERT protein domain structure. Below: Full-length hTERT mRNA, α/β splice variants, drawn as open boxes and approximately to scale. Black arrow indicates open reading frame. Picture adapted from (6).

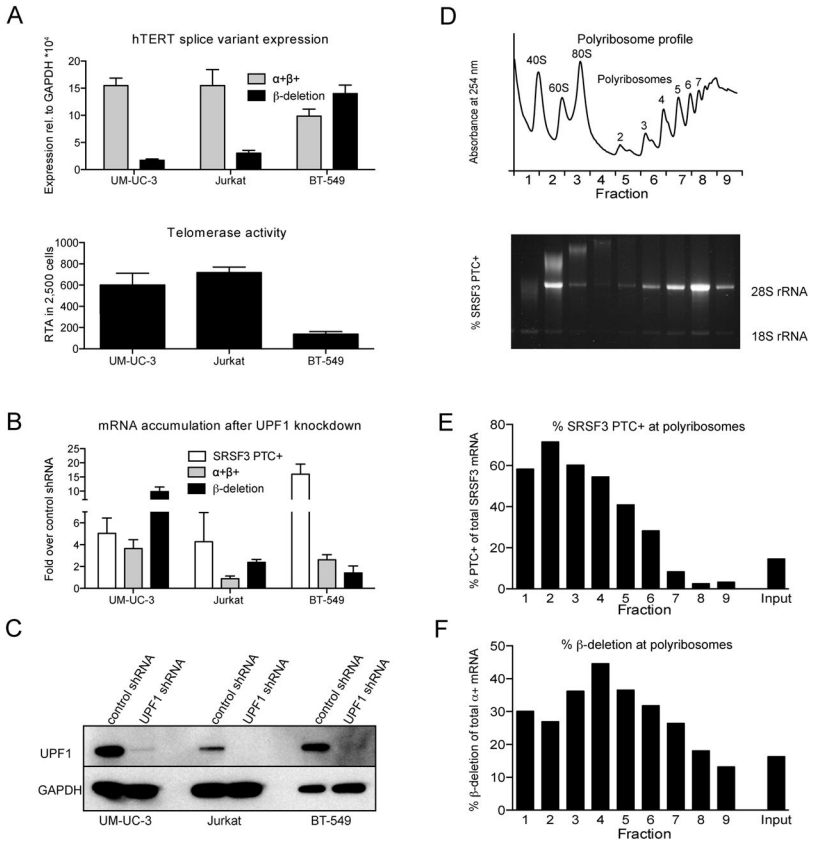

Figure 2. The β-deletion splice variant can escape NMD and is associated with polysomes

A, Top: hTERT α+β+ and β-deletion mRNA splice variant expression in copies/μg RNA, normalized to copies/μg RNA GAPDH mRNA in UM-UC-3, Jurkat and BT-549 cells. Below: Relative telomerase activity (RTA) in 2500 cells/μl. B, Accumulation of hTERT splice variant and SRSF3 PTC+ mRNAs upon UPF1 versus control shRNA knockdown. Error bars are SD from at least 3 experiments. C, Western Blot of UPF1 and GAPDH in cells treated with UPF1 or control shRNAs. D, Top: Representative absorbance profile for RNA separated by velocity sedimentation through a 10–50% sucrose gradient. Positions of 40S, 60S, 80S, and polysomal peaks are indicated. Below: Agarose gel electrophoresis of RNA extracted from each fraction. 28S and 18S rRNAs are indicated. E, Abundance of SRSF3 and hTERT variant mRNA were measured by RT-qPCR and visualized as % SRSF3 PTC+/total SRSF3 and % β-deletion/α+containing hTERT mRNA.

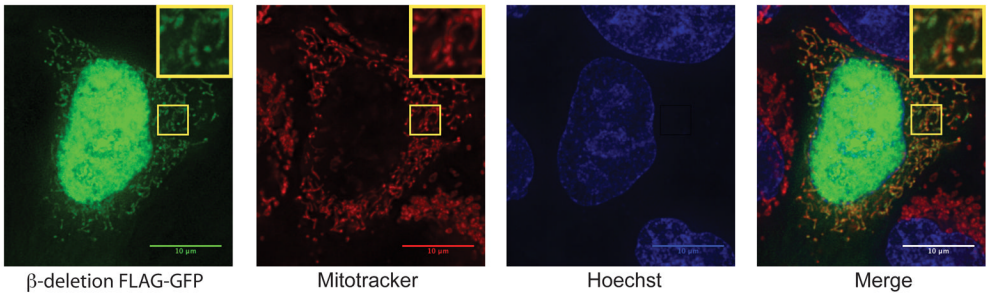

Figure 3. The β-deletion isoform localizes to the nucleus, nucleuolus and mitochondria

β-deletion FLAG/GFP constructs were transfected into HeLa cells and stained with Hoechst 33342 and mitotracker deep red (Molecular Probes).

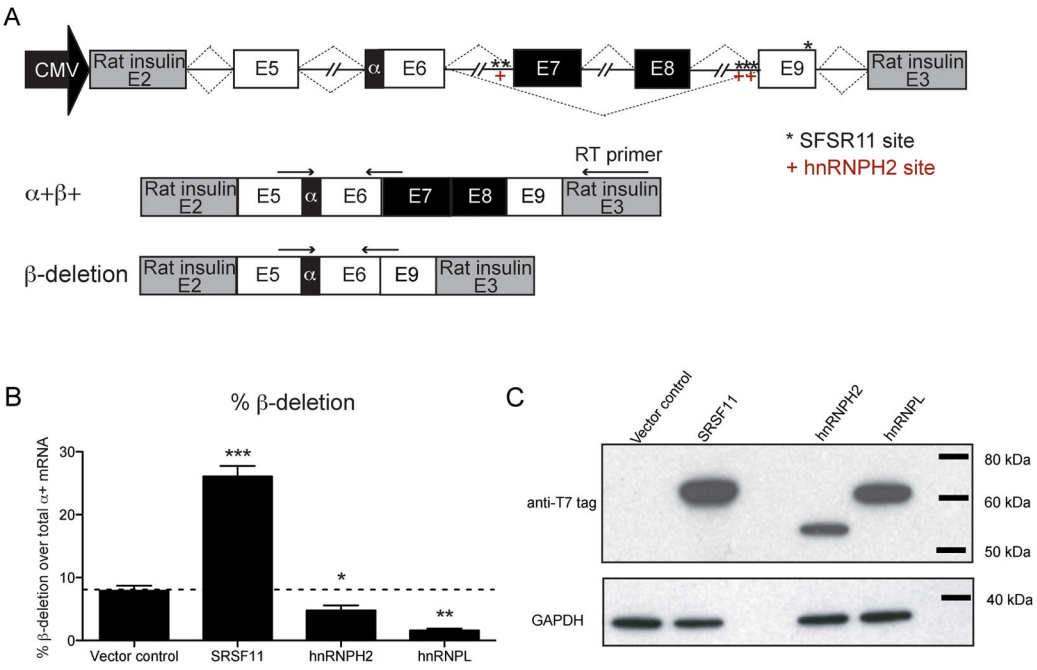

Figure 4. SRSF11, hnRNPH2 and hnRNPL regulate hTERT β-deletion splicing

A, Top: Structure of the pSpliceExpress-hTERT reporter gene. SRSF11 and hnRNPH2 binding sites are indicated with asterisks or plus signs, respectively. Below: qPCR primers for α+β+/β-deletion hTERT variants. RT primer for reporter gene-derived hTERT mRNA anneals to rat insulin exon 3. B, qRT-PCR of hTERT variants from RNA extracted from HEK293T cells co-transfected with either empty plasmid, splicing factor proteins and pSpliceExpress-hTERT. Bars represent mean of % β site exclusion. Error bars are SEM from 3 biological replicates assayed in triplicate. C, Western blot of HEK293T cell extracts extracts confirms overexpression of splicing factors.

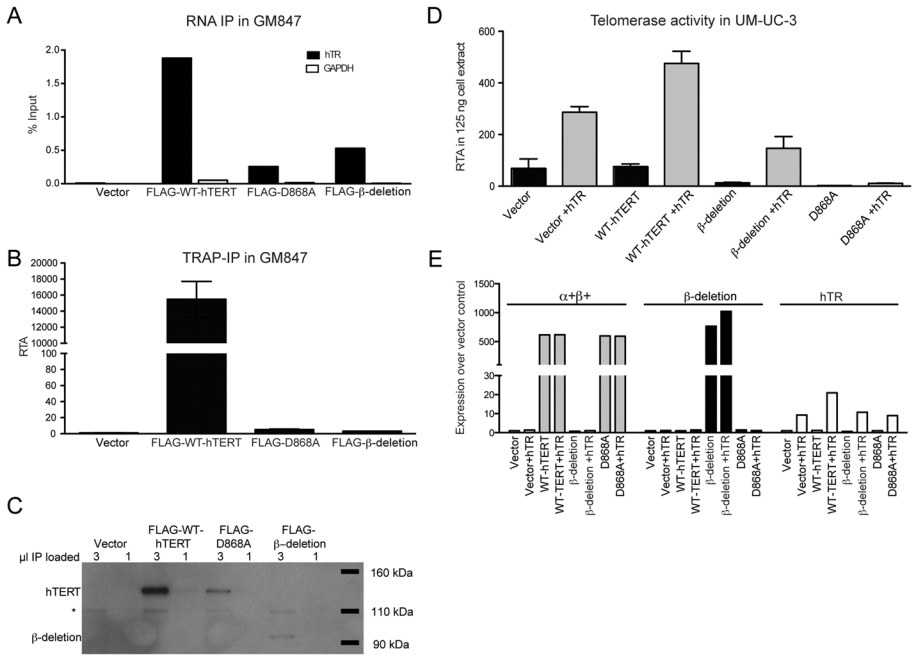

Figure 5. The β-deletion isoform is a dominant-negative inhibitor of telomerase by sequestering hTR

A, hTR RNA associated with FLAG-hTERT constructs in GM847 cells. RNA from a parallel anti-FLAG immunoprecipitates from B was analyzed for presence of hTR and GAPDH RNA by RT-qPCR and repesented as % recovered over input RNA. B, RTA associated with anti-FLAG immunoprecipitates from GM847 cells transduced with indicated lentivirual constructs. C, Western blot of GM847 telomerase RNP immunoprecipitates used in Figure 5B. D, RTA in UM-UC-3 bladder cancer cells transduced with indicated lentiviral vectors. E, RNA from a parallel sample from D was extracted and analyzed for hTR and hTERT variant RNA normalized to GAPDH and vector control. Error bars in B and D represent SD from at least 3 experiments.

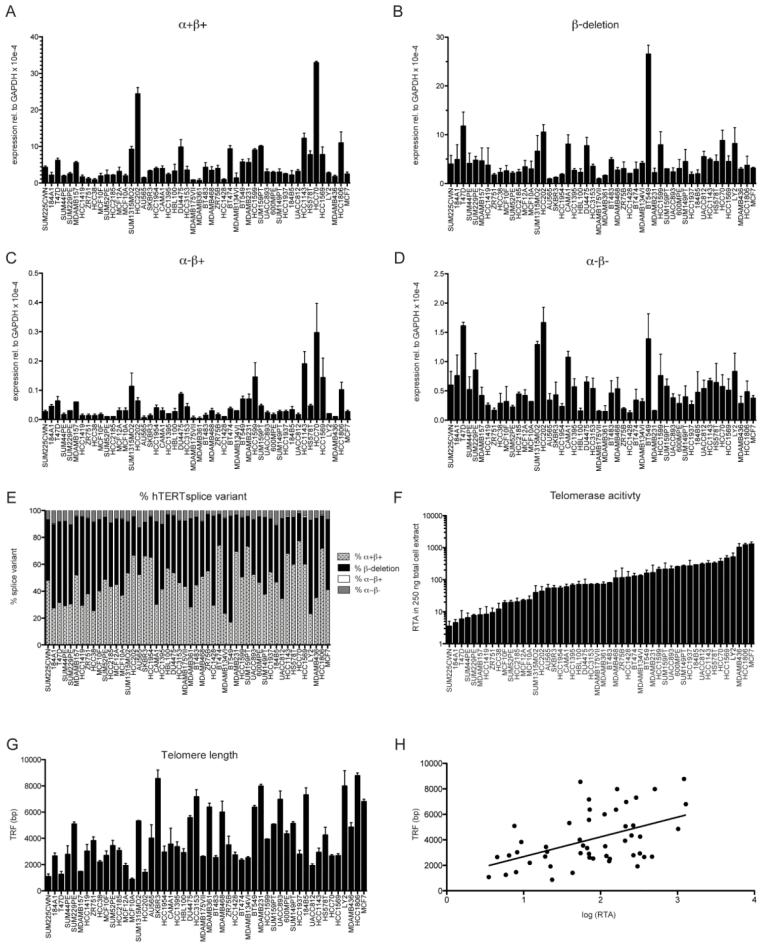

Figure 6. Expression of α/β splice variants, telomerase activity and telomere length in 50 breast cancer cell lines

A–D, Levels of indicated splice variants, expressed as transcript numbers normalized to GAPDH transcript numbers x 104. E, Relative expression of individual α/β hTERT splice variants relative to total amount of hTERT transcripts. F, RTA was measured by RQ-TRAP. G, Modal telomere length was determined by Southern blot from telomere restriction fragments (TRF). H, Regression of log(RTA) and TRF reveals a linear relationship ($r = 0.487$, $P < 0.0001$). Results in A–D, F and G are the average of 2–4 biological replicates and assayed in triplicate reactions (A–D, F); error bars represent SD.

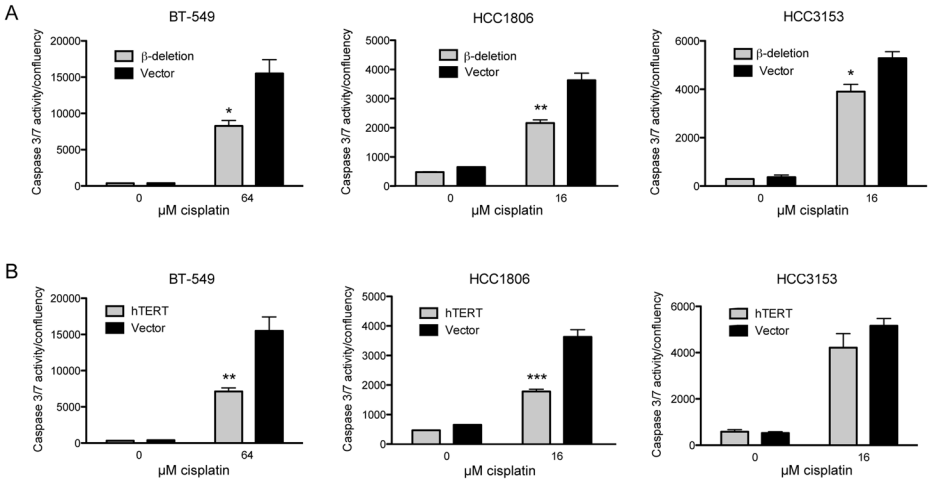

Figure 7. The β-deletion protein protects basal breast cancer cells from apoptosis

BT-549, HCC1806 and HCC3153 that stably overexpressed β-deletion or vector control for less than 2 weeks were treated with the indicated cisplatin concentration for 48 h. Bars represent averaged luciferase activity of caspase 3/7 reporter over cell confluency of 4 replicates; error bars represent SEM.